Antithrombin* Reduces Ischemic Volume, Ameliorates Neurologic Deficits, and Prolongs Animal Survival in Both Transient and Permanent Focal Ischemia

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Background and Purpose—Antithrombin (AT), a glycoprotein belonging to the serpin family, blocks thrombin formation and activity at several steps. Thrombin, beside its relevant role in the coagulation cascade, exerts neurodetrimental effects through the activation of a family of protease-activated receptors, which can be implicated in stroke pathophysiology. The aims of the present study were to evaluate whether AT could reduce brain damage, ameliorate neurologic deficits, and prolong animal survival.

Methods—Two different doses of AT (10 and 30 IU/kg IP) were administered 3 hours, 6 hours, or 3 and 6 hours after an ischemic insult to mice and rats subjected to either transient or permanent focal ischemia. Ischemic volume was evaluated 24 hours or 7 days after the ischemic insult. Neurologic deficits were also scored.

Results—In mice, 10 or 30 IU/kg AT administered twice, at 3 and 6 hours after transient ischemia, and 30 IU/kg AT administered 3 hours only after transient ischemia substantially reduced total ischemic volume, significantly improved neurologic deficits evaluated 24 hours after the insult, and prolonged animal survival. In rats, the same doses given at the same time intervals significantly reduced ischemic volume, evaluated 24 hours after permanent ischemia.

Conclusions—These results indicate that AT remarkably reduces infarct volume, ameliorates neurologic deficit scores, and prolongs animal survival in 2 rodent models of brain ischemia. Taken together, our data suggest that AT, delivered via systemic administration, an easily achievable route of administration and in a clinically useful time window, could represent a new therapeutic strategy to be validated for the clinical treatment of human stroke. (Stroke. 2007;38:3272-3279.)

Key Words: middle cerebral artery occlusion ■ neuroprotection ■ protease-activated receptors ■ stroke

Thrombin, the serine protease generated by prothrombin cleavage, beside having a relevant role in the coagulation cascade, exerts several extravascular effects mediated by the activation of a family of protease-activated receptors (PARs). PARs are G protein–coupled receptors activated by the thrombin-mediated proteolytic cleavage of their N-termini. This cleavage unmasks an amino acid sequence that, by acting as a tethered ligand, activates the receptor.1 Until now, 4 PARs have been described, PAR-1, PAR-2, PAR-3 and PAR-4. Among these different receptors, PAR-1, PAR-3, and PAR-4 can be activated by thrombin, whereas PAR-2 is activated by trypsin.2 Both neuronal and glial cells express PAR-1 mRNA and protein,1 as well as their endogenous ligand precursor prothrombin.3 Interestingly, PAR-1 is found in those brain regions particularly vulnerable to ischemia.4 For instance, PAR-1, which is widely distributed throughout brain tissue,1,5,6 is upregulated after anoxia in hippocampal slice cultures.4 Along with this PAR activation, it has been shown that thrombin levels are increased in the ischemic core zone.7 This can occur because the blood-brain barrier (BBB) becomes more permeable to high-molecular-weight proteins, such as the thrombin precursor prothrombin; the brain itself may be a source of prothrombin;7 and tissue factor, a cofactor of factor VII, which accelerates thrombin generation, has been found in large amounts in the cortex.8 This hypothesis is supported by the fact that prothrombin mRNA is upregulated after cerebral ischemia.7,9 This last evidence, together with the identification of factor X mRNA in the brain, a presence that is required for the conversion of prothrombin to thrombin,10 suggests that thrombin may be released even if the BBB is intact, thus most likely causing brain injury. In addition, both in vivo and in vitro evidence has pinpointed that whereas

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low levels of thrombin seem to be neuroprotective, high thrombin concentrations are deleterious.11 Furthermore, PAR-1 activation is responsible for several neuropathologic effects,6 including neurite retraction,12 cell death in hippocampal cultures and motoneurons,13,14 and potentiation of N-methyl-D-aspartate receptor responses.15 In accordance with these results, mice lacking PAR-1 have a 3.1-fold reduction in infarct volume after transient focal cerebral ischemia.5 Further evidence underlining the role played by thrombin in anoxic brain damage is that intraparenchymal infusion of hirudin, the direct thrombin inhibitor, into the caudate nucleus just after occlusion of the middle cerebral artery mediates neuroprotection.16

Interestingly, antithrombin (AT) is a glycoprotein belonging to the serpin family that blocks thrombin formation and activity at several steps. Furthermore, AT levels decrease in patients who have recovered from ischemic stroke.17 In light of these premises, the aims of this study were to evaluate whether, by using a systemic route of administration and a temporal therapeutic window achievable in human stroke treatment, AT could (1) be effective in reducing the extent of brain damage, (2) ameliorate focal and general neurologic deficits, and (3) improve animal survival after stroke in rodent models of permanent and transient focal ischemia.

To test these hypotheses, AT was intraperitoneally administered to mice and rats subjected to transient or permanent focal ischemia, respectively, at different time points and in doses of 10 and 30 IU/Kg. The intraperitoneal route of administration was chosen because it has been demonstrated that several plasma proteins, including fibrinogen, ferritin, IgG, and albumin, which have a molecular weight analogous to that of AT, can be introduced in the rat via the peritoneal route without altering their subsequent metabolic behavior. Consequently, these proteins can be absorbed quantitatively from the peritoneal cavity, and they distribute thereafter throughout the tissues in the same manner as after intravenous injection.18

Materials and Methods

Experimental Groups
Male CD1 mice (N=116) weighing 25 to 27 g and 59 male Sprague-Dawley rats weighing 250 to 270 g (Charles River) were housed under diurnal lighting conditions. Experiments were performed according to international guidelines for animal research and approved by the animal care committee of the Federico II University of Naples, Italy.

Surgical Procedures

Transient Middle Cerebral Artery Occlusion Model
CD1 mice were subjected to transient middle cerebral artery occlusion (tMCAO) as previously described.19 Anesthesia was induced with 5% isoflurane in a 70% nitrous oxide/30% oxygen mixture and maintained with 2% isoflurane. The right carotid bifurcation was exposed, and the external carotid artery was coagulated distal to the bifurcation. A 5-0 nylon filament was inserted through the external carotid artery stump and advanced into the right internal carotid artery until it blocked the origin of the MCA. After 120 minutes of MCAO, the animals were reanesthetized and the filament was withdrawn to restore blood flow.

Permanent MCAO Model
Permanent MCAO (pMCAO) was performed in Sprague-Dawley rats anesthetized intraperitoneally with chloral hydrate (400 mg/kg).20 Rats were chosen to evaluate whether the neuroprotective effect exerted by AT at 24 hours was maintained over a prolonged period (ie, 7 days). This effect could be better detected in rats because of their increased survival compared with mice. Surgery was performed as previously described.20

Monitoring of Blood Gas Concentration and Cerebral Blood Flow by Laser-Doppler Flowmetry
In some animals, a catheter was inserted into the femoral artery to measure arterial blood gases before and after ischemia (Rapid Laboratory 860, Chiron Diagnostics). Cerebral blood flow (CBF) was monitored in the cerebral cortex ipsilateral to the occluded MCA with a laser-Doppler flowmeter (Periflux System 5000).21 Once a stable CBF signal was obtained, the MCA was occluded. CBF monitoring was continued up to 30 minutes after the end of the surgical procedure, when reperfusion was verified. To verify the effect of AT administration on CBF, in 1 group of animals CBF was monitored from the onset of MCAO to 3 hours after AT administration. No changes in CBF values were found after AT administration in ischemic mice.

Evaluation of Ischemic Volume and of Neurologic Deficit Scores
Mice were decapitated 24 hours after ischemia, and rats, 24 hours or 7 days after ischemia. Ischemic volume was evaluated by 2,3,5-triphenyltetrazolium chloride staining.22 The brains were cut into 500-μm coronal slices with a vibratome (Campden Instrument, 752M). Sections were incubated in 2% 2,3,5-triphenyltetrazolium chloride for 20 minutes and in 10% formalin overnight. The infarcted area was calculated by image analysis software (Image-Pro Plus).23 Total infarct volume was expressed as a percentage of the volume of the hemisphere ipsilateral to the lesion.24

In mice, 24 hours after ischemia, and in rats, 24 hours until decapitation, neurologic function was scored according to 2 scales: a general neurologic scale and a focal neurologic scale.24,25 In the general score, these 6 general deficits were measured: (1) hair conditions (0–2), (2) position of ears (0–2), (3) eye conditions (0–4), (4) posture (0–4), (5) spontaneous activity (0–4), and (6) epileptic behavior (0–12). For each of the 6 general deficits measured, animals received a score depending on the severity of signs. The scores of investigated items were then summed to provide a total general score. In the focal score, these 7 areas were assessed: (1) body symmetry, (2) gait, (3) climbing, (4) circling behavior, (5) front limb symmetry, (6) compulsory circling, and (7) whisker response. For each of these items, animals were rated between 0 and 4 depending on severity. The 7 items were then summed to give a total focal score. AT treatment did not induce any change in neurobehavioral scores in sham-operated animals (data not shown). Ischemic volume, neurologic function, and animal survival were evaluated in a blinded manner.

Experimental Protocol
AT (human AT) saline solution was administered at doses of 10 and 30 IU/kg IP at different time intervals. To exclude any nonspecific protective effect exerted by an inflammatory response in the peritoneum mediated by cytokines and complement, a group of animals was treated with 30 IU/kg AT inactivated by heating at 100°C for 20 minutes and injected 3 and 6 hours after ischemia induction.

Statistical Analysis
Values are expressed as mean±SE. Statistical analysis was performed with 2-way ANOVA. The survival statistical analysis was performed by the log-rank test. Neurologic deficit data were analyzed by the nonparametric Kruskal-Wallis test, followed by the Nemenyi test for the nonparametric multiple comparison. Statistical significance was accepted at the 95% confidence level (P<0.05).
Results

Effect of 10 and 30 IU/kg AT Administration on Infarct Volume and Neurologic Scores After tMCAO in Male Mice

AT at 10 IU/kg administered 3 and 6 hours after tMCAO induced an ∼60% reduction in total ischemic volume compared with vehicle-injected ischemic mice (17.8±9.0% vs 54.4±8.6% of infarct volume compared with the volume of the ipsilateral hemisphere, n=7 in both groups; Figure 1A). AT at 30 IU/kg administered 3 and 6 hours after tMCAO induced an ∼60% reduction in total ischemic volume compared with vehicle-injected ischemic mice (28.3±8.1% vs 54.4±8.6%, n=7 in both groups; Figure 2A). AT at 30 IU/kg administered 3 hours after tMCAO induced an ∼60% reduction in total ischemic volume compared with vehicle-injected ischemic mice (22.9±8.3% vs 54.4±8.6%, n=5 and 7; respectively; Figure 2A).

Interestingly, in all groups treated with AT, infarct volume was significantly reduced in the cerebral cortex but not in the striatum (Figures 1B and 2B). By contrast, when either 10 (n=5) or 30 (n=5) IU/kg AT was administered by single injection 6 hours after MCAO, no significant reduction in ischemic volume occurred, compared with the vehicle-injected ischemic mice (Figures 1A and 2A). Heat-inactivated AT at 30 IU/kg IP injected 3 and 6 hours after ischemia induction did not cause any statistical change in ischemic volume compared with that observed in vehicle-injected ischemic mice (46.96±1.96 vs 54.4±8.6%).

The infarct volume reduction was associated with a significant improvement in general and focal neurologic deficits evaluated 24 hours after tMCAO in comparison with ischemic animals treated with vehicle (Figures 1C, 1D, 2C, and 2D). Intraperitoneal AT administration did not affect PaO2, PaCO2, and pH mean values (data not shown). AT at 30 IU/kg administered 3 hours after ischemia induction did not modify CBF, measured for 3 hours by laser Doppler, compared with vehicle-injected animals (data not shown).

Effect of AT on Mouse Survival

Mice treated with vehicle and subjected to tMCAO displayed a time-dependent mortality, with 10% survival by day 5. The time dependency of the survival rate in untreated mice subjected to tMCAO is in accordance with previous studies.26 Indeed, when mice were treated with systemic 10 or 30 IU/kg

Figure 1. Effect of 10 IU/kg AT on infarct volume and neurologic deficits induced by tMCAO in mice. A, Effect of 10 IU/kg AT on infarct volume induced by tMCAO in mice. B, Effect of AT administered 3 and 6 hours after tMCAO on cortical and striatal ischemic volume. Each column represents the mean±SE of the percentage infarct volume compared with the ipsilateral hemisphere. C, Effect of AT administered 3 hours, 3 and 6 hours, or 6 hours after tMCAO on general and (D) focal neurologic scores. Control animals received vehicle. Ischemic mice were euthanized 24 hours after tMCAO. *P<0.05 vs vehicle-treated ischemic mice, n=5 to 8 animals for each column in panels A–D.
AT at 3 and 6 hours after the beginning of tMCAO, 60% of animals were still alive on day 5, and 30% of animals were still alive on day 6 (Figure 3). The survival rate in mice treated with AT was significantly different from that observed in vehicle-injected mice. The effect of AT on the survival rate of rats subjected to pMCAO was not evaluated, because in this rat ischemic model the mortality rate is very low.

### Effect of AT on Infarct Volume and Neurologic Scores After pMCAO in Male Rats

To validate the protective effect of AT, this drug was tested in another rodent species and in another ischemia model. AT at 10 IU/kg administered 3 and 6 hours after pMCAO induced an \( \approx 50\% \) reduction in total ischemic volume compared with vehicle-injected ischemic rats (16.7\( \pm \)4.4\% vs 30.8\( \pm \)0.5\% of ischemic damage (Figure 4A). By contrast, the focal and general neurologic scores were not reduced (Figures 4C and 4D). The discrepancy between stroke volume reduction and neurologic score observed with the 10 IU/kg AT dose only in rats can perhaps be ascribed to the reduced sensitivity of these tests in rats compared with mice, the animal species in which this method was titrated.

AT at 30 IU/kg administered 3 and 6 hours after pMCAO induced an \( \approx 50\% \) reduction in total ischemic volume compared with vehicle-injected ischemic rats (16.5\( \pm \)2.4\% vs 30.5\( \pm \)0.5\% of ischemic damage (Figure 4B). By contrast, the focal and general neurologic scores were not reduced (Figures 4C and 4D). The discrepancy between stroke volume reduction and neurologic score observed with the 30 IU/kg AT dose only in rats can perhaps be ascribed to the reduced sensitivity of these tests in rats compared with mice, the animal species in which this method was titrated.

### Effect of AT on Survival After tMCAO in Mice

AT at 3 and 6 hours after the beginning of tMCAO, 60% of animals were still alive on day 5, and 30% of animals were still alive on day 6 (Figure 3). The survival rate in mice treated with AT was significantly different from that observed in vehicle-injected mice. The effect of AT on the survival rate of rats subjected to pMCAO was not evaluated, because in this rat ischemic model the mortality rate is very low.

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**Figure 2.** Effect of 30 IU/kg AT on infarct volume and neurologic deficits induced by tMCAO in mice. A, Effect of 30 IU/kg AT on infarct volume induced by tMCAO in mice. B, Effect of AT administered 3 hours, or 3 and 6 hours, after tMCAO on cortical and striatal ischemic volume. Each column represents the mean\( \pm \)SE of the percentage infarct volume compared with the ipsilateral hemisphere. C, Effect of AT administered 3 hours, 3 and 6 hours, or 6 hours after tMCAO on general and (D) focal scores. Control animals received vehicle. Ischemic mice were euthanized 24 hours after tMCAO. *P<0.05 vs vehicle-treated ischemic mice, n=5 to 8 animals for each column in panels A–D.

**Figure 3.** Effect of AT on survival after tMCAO in mice. Ischemic mice received vehicle, 10 IU/kg AT, or 30 IU/kg AT 3 and 6 hours after tMCAO. Survival rate was evaluated every day for 7 days after tMCAO. Each point represents the percentage of animals alive. n=20 animals for each experimental group.
30.8±0.5%, n=5 in both groups; Figure 5A). AT at 30 IU/kg administered 3 hours after pMCAO induced an ~50% reduction in total ischemic volume compared with vehicle-injected ischemic rats (16.2±5.3% vs 30.8±0.5%, n=5 in both groups; Figure 5A). Interestingly, in all groups treated with AT, infarct volume was significantly reduced in the cerebral cortex but not in the striatum (Figures 4B and 5B). The infarct volume reduction was associated with a significant improvement in general and focal neurologic deficits evaluated 24 hours after pMCAO in comparison with ischemic rats treated with vehicle (Figures 5C and 5D).

When either 10 or 30 IU/kg AT was administered in a single injection 6 hours after MCAO, no significant reduction in ischemic volume occurred compared with that observed in vehicle-injected rats (Figures 4A and 5A). Furthermore, when 10 IU/kg AT was administered 3 and 6 hours after pMCAO in male rats, a significant reduction in ischemic volume was observed even 7 days after the insult (18.5±1.9% vs 33.1±2.5%, n=10 in both groups; Figure 6A). The infarct volume reduction was more remarkable in the cerebral cortex than in the striatum (Figure 6B). The reduction in infarct area induced by AT treatment was associated with a significant improvement in both general and focal neurologic deficits in comparison with vehicle-treated rats (Figures 6C and 6D).

**Discussion**

This study presents the first evidence that systemic administration of AT, a thrombin inhibitor, (1) noticeably reduces infarct volume, (2) ameliorates focal and general neurologic deficit scores in 2 different rodent models of MCAO that reproduce human cerebral ischemia, and (3) reduces the mortality rate in mice subjected to tMCAO. Remarkably, AT is effective only if administered 3 hours or 3 and 6 hours after induction of an ischemic insult, a very realistic time period during which its clinical applicability could indeed be conceivable. In accordance with the reduction in ischemic volume and the improvement in neurologic scores, treatment with AT at 10 and 30 IU/kg IP prolonged survival in mice for 7 days after the onset of tMCAO when administered 3 and 6 hours after tMCAO.

Although it has been reported that anticoagulant therapy with low-molecular-weight heparins can be effective in reducing infarct volume in ischemic rodents if administered after the occlusion,27 intriguingly, our results indicate that AT action on brain ischemia does not seem to be ascribed to its powerful anticoagulant effect exerted via thrombin-dependent inhibition of fibrinogen conversion to fibrin. In fact, the results of the present work showed that CBF did not
change in ischemic mice treated with AT 3 hours after ischemia induction. Further support for this hypothesis is that AT, in the permanent ischemia model, was unable to reach the site where clot formation occurred because the artery was electrocauterized. Hence, if the infarct volume reduction had been due exclusively to AT’s anticoagulant property, we would not have found, as we did, any effect in the permanent ischemia model. Further support for this hypothesized mechanism can be found in other experiments performed in an in vitro model of ischemia in hippocampal slice cultures.\textsuperscript{28} Hirudin, a thrombin inhibitor derived from a polypeptide produced by the leech \textit{Hirudo medicinalis},\textsuperscript{29} has been shown to attenuate neuronal death in the CA1 region.\textsuperscript{28} These results support the idea that thrombin, in addition to having an anticoagulant property, has a direct effect on neuronal death. On the other hand, although it has been shown that hirudin attenuates ischemic damage and ameliorates neurologic deficits induced by tMCAO, its protective effect occurred only when it was intracerebrally injected into the caudate nucleus within 75 minutes from the onset of occlusion and 15 minutes before reperfusion.\textsuperscript{16}

Figure 5. Effect of 30 IU/kg AT on infarct volume and neurologic deficits induced by pMCAO in rats. A, Effect of 30 IU/kg AT on infarct volume induced by pMCAO in rats. B, Effect of AT administered 3 hours, or 3 and 6 hours, after pMCAO on cortical and striatal ischemic volume. Each column represents the mean±SE of the percentage infarct volume compared with the ipsilateral hemisphere. C, Effect of AT administered 3 hours, 3 and 6 hours, or 6 hours after pMCAO on general and (D) focal neurologic scores. Control animals received vehicle. Ischemic rats were euthanized 24 hours after pMCAO. *<0.05 vs vehicle-treated ischemic rats, n=5 to 7 animals for each column in panels A–D.

Regarding the possibility that additional mechanisms might be responsible for AT’s action in reducing infarct volume, it must be taken into consideration that AT may exert anti-inflammatory properties, both in vitro and in vivo,\textsuperscript{30–32} by promoting the release of prostaglandin I\textsubscript{2} (prostacyclin) from endothelial cells through its interaction with the cell surface heparin-like glycosaminoglycans.\textsuperscript{32} However, this PGI\textsubscript{2}-mediated anti-inflammatory effect occurs in vivo only when AT is given at much higher doses (100 to 250 IU/kg) than those used in the present study (10 to 30 IU/kg). Such high doses increase the plasma concentration of 6-keto-PGF\textsubscript{1α}, the stable metabolite of PGI\textsubscript{2}.\textsuperscript{31} Indeed, in that report,\textsuperscript{32} it was found that 50 IU/kg AT, a dose 5 times higher than the effective one used in our study, was unable to raise 6-keto-PGF\textsubscript{1α} plasma levels.\textsuperscript{32}

Collectively, all of these data seem to support the concept that extravasal thrombin-mediated mechanisms must be taken into consideration in an effort to unravel the protective effect of AT in reducing the damage after focal ischemia, even though the hypothesis that AT binds to thrombin in the circulation and is cleared from there cannot be presently ruled out. However, it should also be considered that AT diffusion into brain tissue is facilitated during ischemia, not only because the BBB becomes more permeable to high-
molecular-weight proteins, but also because thrombin exerts a disruptive action on the barrier itself. Furthermore, consistent data have been published showing that albumin, a protein with a molecular weight (68 000) similar to that of AT (58 000), crosses the BBB during stroke. Thus, by inhibiting thrombin's action, AT can prevent the thrombin-mediated activation of PAR-1, whose activation leads to neurite retraction, cell death in the hippocampus and motor neurons, and potentiation of N-methyl-D-aspartate receptors. In keeping with AT's mechanism of action, researchers have demonstrated that mice lacking PAR-1 have a 3.1-fold reduction in infarct volume after transient focal cerebral ischemia. In contrast to these cleargcut results, evidence has been provided that PAR-1 activation can prevent apoptosis. Of additional note, the AT doses effective in reducing infarct volume were 8- to 25-fold lower than the ones used to limit disseminated intravascular coagulation in endotoxemic rats and to treat disseminated intravascular coagulation in humans. In conclusion, our preliminary preclinical data can address future clinical studies in the treatment of stroke.

Figure 6. Effect of 10 IU/kg AT on infarct volume and neurologic deficits evaluated 7 days after pMCAO in rats. A, Effect of AT on infarct volume induced by pMCAO. B, Effect of AT on cortical and striatal ischemic volume. Each column represents the mean±SE of the percentage infarct volume compared with the ipsilateral hemisphere. C, Effect of AT administered 3 and 6 hours after pMCAO on general and (D) focal neurologic scores. Neurologic scores were evaluated every 24 hours after pMCAO in rats treated with AT as described. Control animals received vehicle. The white diamonds represent vehicle-treated rats, whereas the black squares represent AT-treated rats. Ischemic rats were euthanized 7 days after pMCAO. *P<0.05 vs vehicle-treated ischemic rats. Statistical analysis was performed with 2-way ANOVA applied to the entire data set, n=5 to 6 animals for each column in panels A-D.

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Disclosures
None.

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